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CONTRIBUTED PAPER

Combining current knowledge of *Cypripedium calceolus* with a new analysis of genetic variation in Italian populations to provide guidelines for conservation actions

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Abstract

The split between conservation science and real-world application is an ongoing issue despite several calls for unification. Researchers are empowered to partially bridge the research-implementation gap by making their findings more accessible. *Cypripedium calceolus* is the most recognizable orchid of the European flora, and is currently facing habitat change and fragmentation, in addition to threats from collectors and illegal traders. Although several studies have focused on the ecological and genetic features of the species, a comprehensive account of how such aspects can be translated into concrete conservation recommendations is still missing. In this study, we describe microsatellite genetic variation in 188 individuals from different Italian populations of *C. calceolus*. Our results indicate the need for immediate conservation action for the most isolated populations in the Central Apennines and north-western Italy. Although our genetic findings are specific to the Italian populations, our aim is to review ecological and population genetic aspects in *C. calceolus* and

their implications for conservation against the existing threats. Therefore, our detailed guidelines for translocation, habitat management and post-translocation monitoring can be used to inform conservation strategies in threatened populations of *C. calceolus* across its range.

KEYWORDS

C. calceolus, conservation genetics, effective population size, lady's slipper orchid, microsatellites, plant translocation, research-implementation gap

1 | INTRODUCTION

Translating scientific findings into practical guidance is a challenging but critical task in conservation biology. Nevertheless, the gap between science and practice represents a common issue in conservation (as reviewed by Knight et al., 2008), particularly in conservation genetics, where the disconnect between research and application spreads from global policy to local implementation (Funk, Forester, Converse, Darst, & Morey, 2018; Hoban et al., 2013; Holderegger et al., 2019; Laikre, 2010; Laikre et al., 2020; Laurance et al., 2012; Shafer et al., 2015; Vernesi & Bruford, 2009). On one side of the gap between science and practice are researchers, whose expertise, often restricted to a specific discipline, often prevents them from effectively translating their findings into comprehensive and jargon-free recommendations (e.g., Britt, Haworth, Johnson, Martchenko, & Shafer, 2018; Hoban et al., 2013; Ottewell, Bickerton, Byrne, & Lowe, 2016). On the other side are practitioners (including local managers to policy makers), who often perceive the few accessible research findings as impractical or unnecessary in the short-term, especially considering time and financial constraints (e.g., Holderegger et al., 2019, but see Cook & Sgrò, 2019a; Taylor, Dussex, & van Heezik, 2017). However, as the failure to incorporate scientific findings into practical conservation guidelines essentially depends on miscommunication and lack of collaboration between researchers and practitioners (Dubois, Gomez, Carlson, & Russell, 2020; Taft et al., 2020), it can be mitigated by providing clear indicators for policy (Hoban et al., 2020), and by combining efforts and expertise on a case-by-case basis (Britt et al., 2018; Cook & Sgrò, 2019b; Hoban, Hauffe, et al., 2013).

In the present study, we focus on one of the most iconic species for plant conservation in Europe, the lady's slipper orchid, *C. calceolus* L. (Orchidaceae; in Italian: "scarpetta di Venere"; Figure 1). Although categorised as a Least Concern (LC) species by the IUCN across its global range (Rankou & Bilz, 2014), *C. calceolus* has disappeared from many parts of Europe and Asia due to habitat fragmentation or habitat change (e.g., stabilisation of substrate, overshading), and depredations of irresponsible collectors (Cribb, 1997; Devillers-Terschuren, 1999; Fay & Taylor, 2015; Goñi, García, & Guzmán, 2015; Jakubska-Busse et al., 2021; Perazza & Decarli, 2020). The species is included in the Annex II of the Habitats Directive of the European Commission (EU, 1992). Extensive research has

FIGURE 1 (a) *Cypripedium calceolus* in the Dolomites, and (b) on the Apennines at PNALM

been conducted on *C. calceolus* in the last decades, encompassing both genetic (e.g., Brzosko, Wróblewska, Tałałaj, & Wasilewska, 2011; Fay et al., 2009; Gargiulo, Kull, & Fay, 2021; Kull & Paaver, 1997; Minasiewicz, Znaniecka, Górniak, & Kawiński, 2018) and ecological aspects (e.g., Davison, Nicolé, Jacquemyn, & Tuljapurkar, 2013; Fay, Feustel, Newlands, & Gebauer, 2018; García, Goni, & Guzman, 2010; Hurskainen et al., 2017; Khapugin, Chugunov, & Vargot, 2017; Kull, 1998; Liu et al., 2021; Nicolé, Brzosko, & Till-Bottraud, 2005; Shefferson, Kull, Tali, & Kellett, 2012), aimed at improving conservation strategies, but also clarifying evolutionary questions (Gargiulo et al., 2019). The most comprehensive studies of *C. calceolus* are those of Devillers-Terschuren (1999) and Kull (1999), and highlight several biological factors that may have contributed to the decline of the species, for example limited pollinator availability (Nilsson, 1979), low fruit-set and low recruitment from seeds, which can be interconnected. The deceptive pollination system is generally associated with low fruit-set (Gill, 1989; Neiland & Wilcock, 1998), and implies that pollinators tend to abandon clumps when they discover sexual deception, promoting outcrossing (Jersáková, Johnson, & Kindlmann, 2006; Tremblay, Ackerman, Zimmerman, & Calvo, 2005; Whitehead, Linde, & Peakall, 2015). Some authors suggest that late-acting self-incompatibility leads to low fruit-set in the species (Pedersen, Rasmussen, Kahandawala, & Fay, 2012 and references therein; Husband & Schemske, 1996), although this aspect has not been thoroughly investigated.

In contrast, longevity (30–100 years; Kull, 1999) promoted by vegetative growth is one of the factors favouring population persistence and stability (Brzosko, Wróblewska, & Ratkiewicz, 2002) and counteracting the effects of genetic erosion (Gargiulo et al., unpublished). In fact, the clonal shoots (i.e., ramets) emerging from the same rhizome over the years are responsible for most of the fluctuations in the apparent number of individuals in a population, whereas the number of genetic individuals generated by sexual reproduction (i.e., genets) is usually stable (Kull, 1995). Furthermore, the relationship between the number of ramets and the number of genets also affects the estimation of the effective population size, N_e, that is the ideal population size generally used to predict the strength of genetic erosion (e.g., Frankham, 1995; Waples, 2005). In *C. calceolus*, N_e may be <30% of the genets in the population and <5% of the number of emerging ramets (Gargiulo et al., unpublished), implying that the difference between the number of ramets and the number of genets must not be disregarded when examining genetic diversity in this species.

Such ecological and genetic considerations demonstrate that conserving *C. calceolus* requires expertise from different fields, including pollination biology, demography, genetics, microbiology, seed biology, horticulture, and habitat management in general, as observed for other orchid species (Calevo et al., 2021; Fay, 2018; Gale, Fischer, Cribb, & Fay, 2018; Phillips, Reiter, & Peakall, 2020). Moreover, the need for synergy among the many interested parties, for example, members of the public, conservation and natural history societies, local universities and regional and national conservation bodies, can also hinder conservation actions due to the difficulty in coordinating efforts.

Among the most successful recovery plans for *C. calceolus*, that executed in England (Corkhill, 1996; Ramsay & Stewart, 1998; Fay & Taylor, 2015; see also Redshaw, 2019) is a renowned example. In vitro propagated plants (Box 1), reintroduced in the wild in the last 20 years, have now also acquired mycorrhizal partners (Fay et al., 2018; see Shefferson, Weiß, Kull, & Taylor, 2005 and Shefferson et al., 2007 for studies about mycorrhizal specificity in slipper orchids). Moreover, the species has been propagated on a large scale by the commercial nursery Anthura in the Netherlands, where the concerted actions of different stakeholders have provided funds for the restoration of populations in the wild (Gale et al., 2018). The same nursery was commissioned by the Swiss Orchid Foundation to propagate plants for translocation using local seeds in Switzerland, where some populations have been shockingly reduced from thousands to fewer than five individuals in one night (De Jong & Sprunger, 2020). The translocation experiment achieved a success rate ~80% of the initial 3,000 plants reintroduced in 44 sites (Cribb, 2020; De Jong & Sprunger, 2020). A similar translocation project is currently being developed in Germany. A lesson learnt from these projects is that, unless specialist knowledge and infrastructure are available, the leap from in vitro to in situ may never be successful.

In Italy (Bartolucci et al., 2018; GIROS, 2016), *C. calceolus* occurs in the Alpine mountain range in Northern Italy, being particularly abundant in the Eastern Alps (Bonazza, Caniglia, Moraldo, & Rossi, 2004; Perazza & Decarli, 2020; Perazza & Lorenz, 2013) and becoming progressively rarer towards the west (e.g., Bovio, 2014; Martini, Bona, Federici, Fenaroli, & Perico, 2012). In the Central Apennines, two populations occur within the Maiella National Park (hereafter indicated as PNM; Conti, Ciaschetti, Di Martino, & Bartolucci, 2019) and the Abruzzo, Lazio and Molise National Park (hereafter indicated as PNALM; Conti & Bartolucci, 2015). According to the IUCN criteria, *C. calceolus* is categorized as Least Concern (LC) on the

BOX 1 In vitro seed germination of *Cypripedium calceolus* in England

In vitro seed germination of *C. calceolus* has been intensively investigated in England, with the aim of rescuing the last wild population (represented by a single plant) and of establishing self-sustaining populations at other sites where it previously occurred. In particular, the In Vitro *Research group* at the Royal Botanic Gardens (RBG), Kew has been working to refine in vitro germination in the absence of mycorrhizal partners (asymbiotic germination), and to determine and leverage mycorrhizal partners in the wild plant for symbiotic in vitro germination.

Asymbiotic germination. This was achieved by germinating seeds in vitro by using the "green-pod" method reported by Ramsay and Stewart (1998), which was developed from the technique pioneered by Malmgren in Sweden (Cribb, 2020; Malmgren, 1996). Green capsules (45–55 days of maturity after pollination) were collected, and seeds were germinated in vitro over a four- to six-month period. After the development of protocorms, seedlings with well-developed root systems and shoots were bagged up and chilled at 4°C for 4 weeks before being potted up. The green-pod method is used to produce seedlings every year for reinforcement of the wild population and provide material for use in establishing reintroduction sites. However, natural recruitment of seedlings still appears to be a potential problem. Changes in weather, especially dry or wet periods, makes collecting of green capsules of the right maturity difficult in some years, meaning that fewer plants are available. Nevertheless, in the past few years, plants of *C. calceolus* have been successfully reintroduced to several sites in northern England (Cribb, 2020; Fay & Taylor, 2015).

Symbiotic germination. Root samples were collected from one of the naturally recruited seedlings close to the last naturally occurring plant in the UK wild site. Isolation of pelotons, preparation of in vitro fungal cultures and identification of fungal accessions (i.e., Operational Taxonomic Units, OTUs) using Internal Transcribed Spacer sequencing were completed following the protocols as described by Yokoya et al. (2015). Roots collected from seedling from the wild site yielded several fungal OTUs, especially two putative mycorrhizal fungi belonging to the Ceratobasidiaceae clade. Non-mycorrhizal fungal orders that are commonly found in orchids such as Helotiales and Hypocreales were also abundant in the seedling roots. *Cadophora* sp. (Helotiales) was the most dominant non-mycorrhizal fungus (Figure 2). *Ceratobasidum albasitensis* and other unidentified OTUs of Ceratobasidiaceae were used for in vitro symbiotic seed germination trials using mature seeds from the last wild plant and three other individuals. An uncultured Ceratobasidiaceae isolate initiated seed germination of seeds collected from the wild plant. However, use of these putative mycorrhizal isolates failed to induce seed germination. The developed protocorms failed to progress into fully symbiotic seedlings. Further sampling of roots from wild-collected samples from mainland Europe may help identify compatible mycorrhizal fungi for successful symbiotic seed germination. Symbiotic seedlings are likely to be better suited for reinforcement/reintroduction (Rafter, Yokoya, Schofield, Zettler, & Sarasan, 2016; Yokoya et al., 2021), especially for species like *C. calceolus* as suitable mycorrhizal associates may not be present at (re-) introduction sites.

Italian Red List (Rossi et al., 2013, 2016). However, the conservation status of the latter populations is particularly concerning, because of their small sizes (~25 ramets in PNM and ~720 ramets in PNALM). These populations have recently been among the targets of the Floranet Life Project, which has implemented several conservation actions (Di Martino, Conti, Bartolucci, Ciaschetti, & Di Cecco, 2016; Box 2). As the Apennine populations of *C. calceolus* are the southernmost in Europe (but note that a disjunct population was recently discovered in Algeria; Nemer, Rebbas, & Krouchi, 2019), they may be considered vulnerable "edge populations" (but see García et al., 2010). This hypothesis is supported by the recent findings of elevation range shift towards higher elevations for *C. calceolus* in the Alpine range (Geppert et al., 2020; Perazza & Chini, 2020). However, species distribution/ecological niche modeling includes the Apennines in the area/niche of current and future suitability for the species, although possibly subject to range contraction (Gargiulo et al., 2019; Kolanowska & Jakubska-Busse, 2020).

The main aim of the present study is to review ecological and population genetic aspects in *C. calceolus*, to provide precise guidelines for future conservation actions against the threats to the species. To achieve this, we describe genetic diversity and differentiation in several

BOX 2 *Cypripedium calceolus* in the Central Apennines: observations, in situ and ex situ conservation interventions

Cypripedium calceolus is included in the LIFE FLORANET project "LIFE15 NAT/IT/000946," the main objective of which is to protect and to improve the conservation status of seven vascular plants listed under the Habitats Directive 92/43/EEC, occurring in Natura 2000 areas in the Central Apennines. During the project, started in 2016 and still in progress, ecological, floristic and taxonomic aspects have been also investigated (Bartolucci & Conti, 2019; De Castro et al., 2020; Di Musciano et al., 2020). Several concrete actions have been carried out for in situ and ex situ conservation and management of *C. calceolus*. The first action was aimed at obtaining information about the exact distribution and the conservation status of the populations of *C. calceolus*. For each site, information about the localization, surface area, population size, habitat, main pressures/threats and conservation measures were evaluated. Each population traced was analyzed according to the monitoring protocol written by SBI (Italian Botanical Society) and ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale) for the third report under Article 17 of the Habitats Directive (Ercole, Giacanelli, Bacchetta, Fenu, & Genovesi, 2016). Field investigations showed that the population in the PNM consists of 25 ramets and is currently decreasing, whereas in PNALM the species is in a better state of conservation and has ~720 ramets (five sub-populations). Some in situ interventions with the aim of reducing the pressures on single individuals and/or population of *C. calceolus* have been made through 1) construction of small fencing structures (five in PNM, ten in PNALM), to protect the plants from domestic (especially horses) and wild animals (deer, hares, etc.), and from poaching; 2) installation of wooden fences in PNALM with the aim of stopping the passage of domestic animals and humans (350 m) in the habitats that are best known by people; and 3) interventions of small natural engineering to reduce erosion and removal of gravel accumulation along the Scerto river in PNALM. Finally, in PNALM, in places where tree canopy was shading plants of *C. calceolus*, some experimental silvicultural interventions have been carried out. They consisted of prudent, one-off and ongoing interventions, selecting only some small trees or some branches that were shading the populations, and modulating them at different times where needed, to obtain semi-shading and not a clear-cut. Monitoring such interventions will be crucial to understanding their effectiveness and to compare them with "non-intervention" situations. Considering the general decrease in fruiting (data from before the beginning of the project), we carried out artificial pollinations by withdrawing pollen from one individual with a toothpick and transferring it to another flower. In PNM, five fruits were obtained from the five (out of seven) hand-pollinated flowers in 2017, and one fruit from three hand-pollinated flowers in 2018; in PNALM, we obtained 38 fruits from the 65 hand-pollinated flowers in 2017, two fruits from seven hand-pollinated flowers in 2018, 17 fruits from 22 hand-pollinated flowers in 2019 and ten fruits from 14 hand-pollinated flowers in 2020. Concerning the ex situ actions, we collected seeds in different populations from PNALM and PNM, following international collection protocols (ENSCONET, 2009). Some of the collected seeds were stored at the Maiella seed bank (Di Cecco et al., 2020), and some were used to produce new plants by in vitro germination techniques at the Tuscia Germplasm Bank (Figure 3). For *C. calceolus*, two germination protocols were applied using both mature and immature seeds. Seedlings were obtained only using immature seeds of PNALM, following the green-pod protocol of Malmgren (1996). Currently, the seedlings (~100) are growing at the Tuscia Germplasm Bank. In the next steps, the seedlings will be placed in soil in pots, and will be grown in the nurseries of PNM. Mature plants will be used to reinforce the existing populations in PNM and PNALM. The development of the species is slow, and therefore the restocking of the plants will be possible only in about 3 years, during the After-LIFE project phase. Although at the end of the LIFE FLORANET project the achieved demographic increase for the target species will be lower than expected, the final improvement of their conservation status will be stronger than initially planned.

populations of *C. calceolus* from the Italian range using microsatellite markers, and we translate our conservation genetic findings and the current knowledge of the species into conservation recommendations. Although some of these recommendations will be population-specific, our synthesis linked to the ecology and the population genetics of *C. calceolus* may inform strategies in other populations throughout the range of the species.

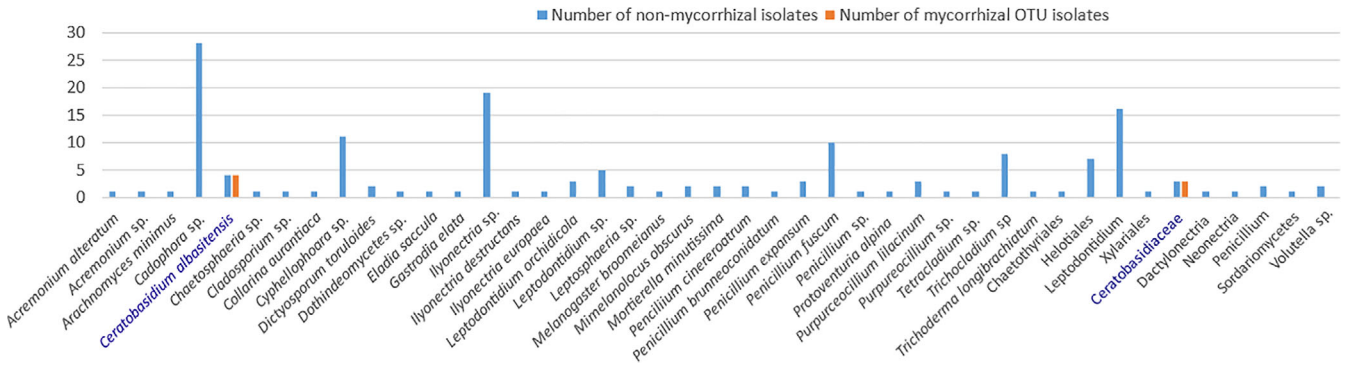

FIGURE 2 (In Box 1). Putative mycorrhizal and non-mycorrhizal fungi identified from the seedling of British *Cypripedium calceolus* (colored text shows putative mycorrhizal fungi)

2 | MATERIALS AND METHODS

2.1 | Population sampling and nSSR genotyping

Populations of *C. calceolus* sampled for the present study are reported in Table 1 and include the populations analyzed in Gargiulo et al. (2019). In addition, the population in the Pesio Valley, isolated at a location in the Western Alps of Italy, was sampled during Summer 2018. This population, which was plundered for herbarium specimens at the beginning of 1900s (Isaja & Dotti, 2003), was re-discovered in 1991, with three sites at different elevations (Gallino, Ostellino, & Re, 1993). Since then, the number of individuals has had a significant decline, despite its secluded location. Between 2017 and 2019, some conservation strategies were implemented, including mitigating soil erosion in the hills surrounding the valley, artificial pollination, in vitro seed germination, and rhizome translocation to the botanical alpine garden. The most conspicuous core of the Pesio Valley population includes ~100 ramets; at the time of sampling, about half of the individuals were flowering.

At all sampling sites, leaf fragments were collected from individuals located at least 20 cm apart whenever possible, to exclude ramets emerging from the same rhizome. In Table 1, we make the distinction between "sampling site" and "population," with the latter indicating groups of sampling sites at short distances (<5 km) or characterized by spatial continuity. Such a flexible designation is used throughout the analyses to account for ongoing gene flow and to prevent prior assumptions about genetic groups which may bias interpretations (Balloux & Lugon-Moulin, 2002). In fact, gene flow can both occur by wind-dispersal of the microscopic seeds of *C. calceolus* and by pollination mediated by solitary bees (e.g., species of *Andrena* Fabricius and *Lasioglossum* Curtis, probably through sexual deception; Antonelli, Johan Dahlberg, Carlgren, & Appelqvist, 2009; Przybyłowicz et al., 2012; Braunschmid et al., 2017), which are capable of travelling limited distances (usually <1 km; Gathmann & Tscharntke, 2002; Hofmann, Fleischmann, & Renner, 2020; potentially reaching longer distances; Zurbuchen et al., 2010; Cane & Love, 2019).

DNA extraction and microsatellite genotyping followed Gargiulo et al. (2019), except for the DNA samples from the Pesio Valley, which were extracted using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, Georgia, US). In addition to the previously employed 11 nuclear microsatellite loci (Gargiulo et al., 2019), we analyzed variation for the locus Ccal_50 (Minasiewicz & Znaniecka, 2014). We performed allele calling in GeneMapper v5.0 (Applied Biosystems) and used MicroChecker v2.2.3 (van Oosterhout, Hutchinson, Wills, & Shipley, 2004) with 1,000 randomizations to inspect the data set for scoring errors.

2.2 | Genetic diversity and differentiation

We used the *R* v3.6.3 (R Development Core Team, 2013) package *poppr* v2.8.3 (Kamvar, Brooks, & Grünwald, 2015; Kamvar, Tabima, & Grünwald, 2014) to assess the occurrence of putative clones, represented by identical multilocus genotypes (MLGs), and to detect deviations from Hardy–Weinberg proportions with 1,000 permutations. We kept one representative multilocus genotype for each group of putative clones and used the obtained data set in the subsequent analyses to avoid the influence of vegetative reproduction on the estimation of genetic diversity and differentiation.

We calculated observed heterozygosity (H_O), (unbiased) expected heterozygosity (uH_E) and private alleles in GenAlEx v6.5 (Peakall & Smouse, 2006). We also

TABLE 1 Details of the populations of *Cypripedium calceolus* analyzed in the present study

Sampling site[a]	Population[a]	Estimated number of ramets[a]	Sample size[b]	H_O (SE)		uH_E (SE)		Private alleles[c]		Allelic richness[d]		N_e (CI)[e]	F_{IS}	
				Per sampling site	Per population	Per sampling site	Per population	Per sampling site	Per population	Per sampling site	Per population		Per sampling site	Per population
Maiella	Maiella National Park—PNM, Central Apennines	~25	11 (14)	0.557 (0.089)	0.557 (0.089)	0.468 (0.073)	0.468 (0.073)	—	—	2.43	2.56	10.8 (2.9–321.1)	−0.201	−0.201
Torrente Scerto	Abruzzo, Lazio and Molise National Park—PNALM, Central Apennines	~720	20 (21)	0.520 (0.074)	0.513 (0.068)	0.528 (0.068)	0.513 (0.072)	3	4	3.02	3.81	16.1 (8.6–31.7)	0.016	0.063
Rifugio della Liscia			19 (22)	0.507 (0.066)		0.545 (0.074)		1		3.24			0.071	
Brenta Valley	Brenta Dolomites (Adamello-Brenta Natural Park)	>10,000	20	0.629 (0.053)	0.650 (0.045)	0.660 (0.053)	0.677 (0.050)	2	13	3.90	5.06	226.6 (114–1,390.9)	0.048	0.040
Nardis			19	0.662 (0.045)		0.696 (0.045)		3		4.07			0.050	
Tovel			21	0.663 (0.056)		0.674 (0.056)		—		4.12			0.018	
Algone			18	0.644 (0.050)		0.646 (0.052)		1		3.84			0.004	
Sporeggio			10 (11)	0.658 (0.068)		0.661 (0.055)		—		3.92			0.005	
Goletto Gaver	Gaver, Adamello Park, Lombardy	~300	8 (12)	0.573 (0.071)	0.551 (0.065)	0.554 (0.053)	0.580 (0.052)	—	—	3.05	4.19	95.4 (15.2-infinite)	−0.036	0.051
Piana del Gaver			5 (12)	0.517 (0.083)		0.569 (0.066)		—		3.58			0.101	
Pesio Valley	Pesio Valley, Piedmont, Western Alps	~100	37 (48)	0.609 (0.063)	0.609 (0.063)	0.610 (0.059)	0.610 (0.059)	2	2	3.45	4.03	3.1 (2.3–5.1)	0.002	0.002

Abbreviations: CI, 95% confidence interval; F_{IS}, inbreeding coefficient; H_O, observed heterozygosity; uH_E (unbiased), expected heterozygosity; N_e, effective population size.

[a]Rationale: we compared genetic diversity indices obtained by (a) considering single sampling sites as isolated demes and (b) by considering sampling sites at short distances as one population (as sites at short distance are connected by gene flow via pollen and seeds). The estimated number of ramets refers to the entire population (not limited to the sampling sites).

[b]Sample sizes for genetic analysis in parentheses indicate the initial sample size (putative clones included).

[c]Private alleles refer to the data set analyzed in this study. All private alleles at PNALM and Pesio Valley are otherwise shared with Eurasian populations. Only three alleles in the Brenta Dolomites population have not been found elsewhere in Eurasia (Gargiulo et al., 2019).

[d]Allelic Richness based on the minimum sample size (5 individuals for "sampling site" analysis and first and 9 individuals for "population analysis"), as computed in FSTAT.

[e]N_e computed with the LD method; threshold to screen out rare alleles (p-crit) equal to 0.02, CI estimated by jackknife.

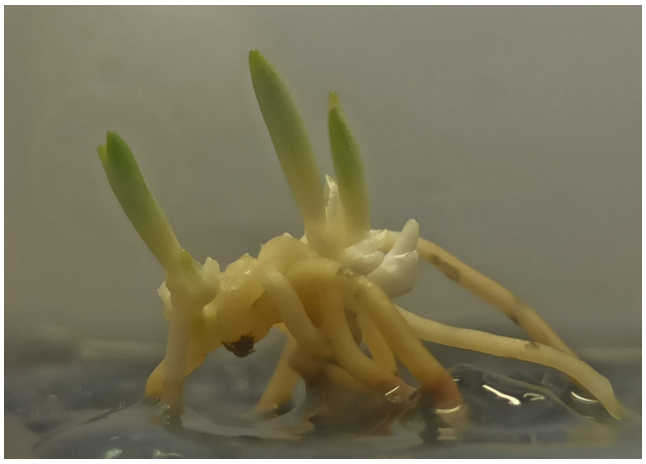

FIGURE 3 (In Box 2). Seedling of *Cypripedium calceolus* germinated at the Tuscia Germplasm Bank

compared private alleles found in the present data set with private alleles found elsewhere in Eurasia (Gargiulo et al., 2019). We used FSTAT v2.9.3.2 (Goudet, 2001) to compute allelic richness and the inbreeding coefficient, F_{IS} (Weir & Cockerham, 1984). We computed genetic diversity parameters considering both the sampling site and the population subdivision, as defined in Table 1.

We used multiple approaches to investigate population structure and genetic differentiation. In Arlequin v3.5 (Excoffier & Lischer, 2010), we performed a hierarchical analysis of molecular variance (AMOVA; Weir & Cockerham, 1984; Excoffier, Smouse, & Quattro, 1992; Weir, 1996) to test genetic structure within and among groups of sampling sites at short distances and computed pairwise population (sampling site) differentiation. For both analyses, we used the sum of squared size difference (RST) as a distance method, with 1,000 permutations. In Structure v2.3.4 (Pritchard, Stephens, & Donnelly, 2000), we inferred genetic structure through Bayesian clustering, using the Admixture model and without using sampling sites as a prior assumption. We tested K (number of genetic clusters) values ranging from 1 to 10, with 10^6 burn-in and 10^6 MCMC replicates, with 30 iterations. The Structure analysis was run using StrAuto (Chhatre & Emerson, 2017) and GNU parallel (Tange, 2011). We evaluated the most likely K both with the LnPr($X|K$) method (Pritchard & Wen, 2003) and with the ΔK method (Evanno, Regnaut, & Goudet, 2005), using the online tool Structure Harvester (Earl & vonHoldt, 2012), and summarized the results in CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). As the method implemented in Structure assumes panmictic populations (Pritchard et al., 2000), we also carried out a Discriminant Analysis of Principal Components (DAPC) in the *R* package *adegenet* v2.1.1 (Jombart, 2008; Jombart & Ahmed, 2011), which is more suitable for partially clonal populations (Jombart, Devillard, & Balloux, 2010). We used the cross-validation method to infer the appropriate number of components (PCs) to retain for the Principal Components Analysis (PCA). A scatter plot of the first two PCs was produced to describe genetic differentiation.

2.3 | Estimation of the effective population sizes and bottleneck tests

We estimated contemporary effective population sizes (N_e) for the Italian populations of *C. calceolus* using the software NeEstimator v2.1 (Do et al., 2014). We employed the linkage disequilibrium method LDN_e; Hill, 1981; Waples & Do, 2008) implemented in the software, screening out alleles with a frequency (p-crit) < 0.02 (Waples & Do, 2010; Waples, Larson, & Waples, 2016) and computing confidence intervals by jackknifing over individuals (Do et al., 2014; Jones, Ovenden, & Wang, 2016). The linkage disequilibrium method assumes isolated populations, which is realistic for the populations at Maiella, PNALM and Pesio Valley, but less realistic for the populations located in spatial continuity (e.g., Brenta Dolomites). Further caveats regarding the estimation of N_e for our data set are (a) the limited number of microsatellite markers we employed and the small sample sizes for the population at Maiella (11) and Gaver (12), which may give less robust confidence intervals for N_e; (b) the biology of the species and the sampling strategy, as overlapping generations and mixed-age adult samples may produce biased estimates of N_e (Waples, Antao, & Luikart, 2014). The extent of such potential biases, however, are compensated by the benefits of obtaining even approximate estimates for N_e for conservation decisions (Frankham, 2021; Garner, Hoban, & Luikart, 2020; Hoban et al., 2021; Laikre et al., 2021; Luikart et al., 2021).

We used the software Bottleneck v1.2 (Piry, Luikart, & Cornuet, 1999) to detect heterozygosity excess possibly due to a recent reduction in population size, under the three mutation models implemented in the software, with default parameters (Infinite Allele Model, IAM; Stepwise Mutation Model, SMM; and Two-Phase Model, TPM, which is considered as the most appropriate model for microsatellites; Di Rienzo et al., 1994). To assess the occurrence of a significant number of loci with heterozygosity excess, we performed the sign test and the Wilcoxon signed-rank test and verified the occurrence of a shift in the allele frequencies with the qualitative test of mode-shift (Cornuet & Luikart, 1996; Luikart, Allendorf, Cornuet, & Sherwin, 1997; Luikart &

Cornuet, 1998; but see Peery et al., 2012, for a review of the typical pitfalls of Bottleneck tests).

3 | RESULTS

3.1 | Genetic diversity and differentiation

We genotyped 218 individuals of *C. calceolus* for 12 microsatellite loci (File S1); no evidence of scoring errors was found in Micro-Checker. We excluded 30 potential clones identified with *poppr*, all detected in individuals from the same sampling localities. The final data set with no replicated genotypes included 188 individuals.

Population-specific deviations from the Hardy–Weinberg proportions were found in Pesio Valley (File S2, Figure S1), mostly associated with positive F_{IS} values and missing data (File S2, Tables S1 and S2; Figure S2). Most of the other deviations from the Hardy–Weinberg proportions depended on deme subdivision (see File S2, Figure S1). Observed heterozygosity ranged from 0.51 (Rifugio della Liscia) to 0.66 (Tovel), and uH_E heterozygosity ranged from 0.47 (Maiella) to 0.70 (Nardis). When combining sampling sites across short geographical distances, H_O ranged from 0.51 (PNALM) to 0.65 (Brenta Dolomites), and uH_E ranged from 0.47 (Maiella) to 0.68 (Brenta Dolomites) (Table 1). Private alleles were found at Scerto, Rifugio della Liscia, Brenta, Nardis, Algone and Pesio Valley sampling sites. However, when comparing our data set with the Eurasian data set (Gargiulo et al., 2019), only two alleles at Nardis and one at Brenta Valley were truly private. Allelic richness followed the same trend as uH_E, with the lowest value (~2.5) found at Maiella and the highest values (~5.0) found in the Brenta Dolomites (Table 1; Figure 4). Inbreeding coefficients, F_{IS}, ranged from negative to positive values, differentially displayed in different loci and populations (File S2, Tables S1 and S2); values averaged across loci were mostly >0, except in Maiella (−0.20) and Goletto Gaver (−0.04) (Table 1).

Results of the hierarchical AMOVA indicate most of the variation (91.3%) partitioned within sampling sites, whereas 7% of the variation was found within groups of sampling sites at short distances (File S2, Table S3). The highest pairwise differentiation was found between the individuals in Maiella population and the ones in Gaver (~0.3), whereas in general pairwise differentiation was low (<0.15; File S2, Figure S3). Negative pairwise differentiation values were detected for a few pairs corresponding to sampling sites at short distances (Scerto-Rifugio della Liscia, Brenta-Tovel, Tovel-Algone, Algone-Sporeggio, Nardis-Sporeggio) and for the pair Tovel-Piana del Gaver, located at greater distances;

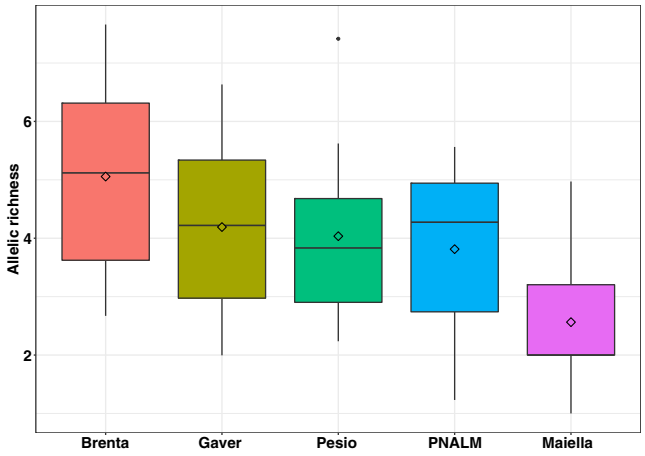

FIGURE 4 Allelic richness for the populations of *Cypripedium calceolus* as estimated in FSTAT. Values averaged over loci are indicated by diamonds

most of the pairwise differentiation tests between pairs at short distances were also non-significant (p-value >0.05) (File S2, Figure S2).

The modal value for ΔK (Evanno method) was associated with $K = 3$, and the pre-plateau value in the L(K) plot was $K = 4$ (File S2, Figure S4). The three genetic clusters for $K = 3$ correspond to the groups including (1) the Apennine populations (Maiella and PNALM), (2) the Brenta Dolomites and Gaver populations, (3) the Pesio Valley population (Figure 5). When considering $K = 4$, Maiella and PNALM form separate genetic clusters. From the Structure analysis, Pesio Valley represents the most admixed population, where many individuals differentially belong to one of the clusters, even when considering $K = 2$ for comparison (Figure 5).

After the cross-validation procedure carried out in adegenet, we retained 48 PCs. The DAPC revealed low differentiation among populations (Figure 6), especially among the individuals from the Brenta Dolomites. The most differentiated individuals were the ones occurring in the Maiella population, which formed an isolated cluster. Other discrete clusters were those including individuals from Pesio Valley and including individuals from PNALM and Gaver (Figure 6).

3.2 | Estimation of the effective population sizes and bottleneck tests

Contemporary N_e ranged from 3 (95% CI: 2.3–5.1) in Pesio Valley, to ~227 (95% CI: 114–1,391) in the Brenta Dolomites (Table 1). In the Gaver population, we detected an infinite upper interval for the 95% CI, probably related to the small number of individuals sampled.

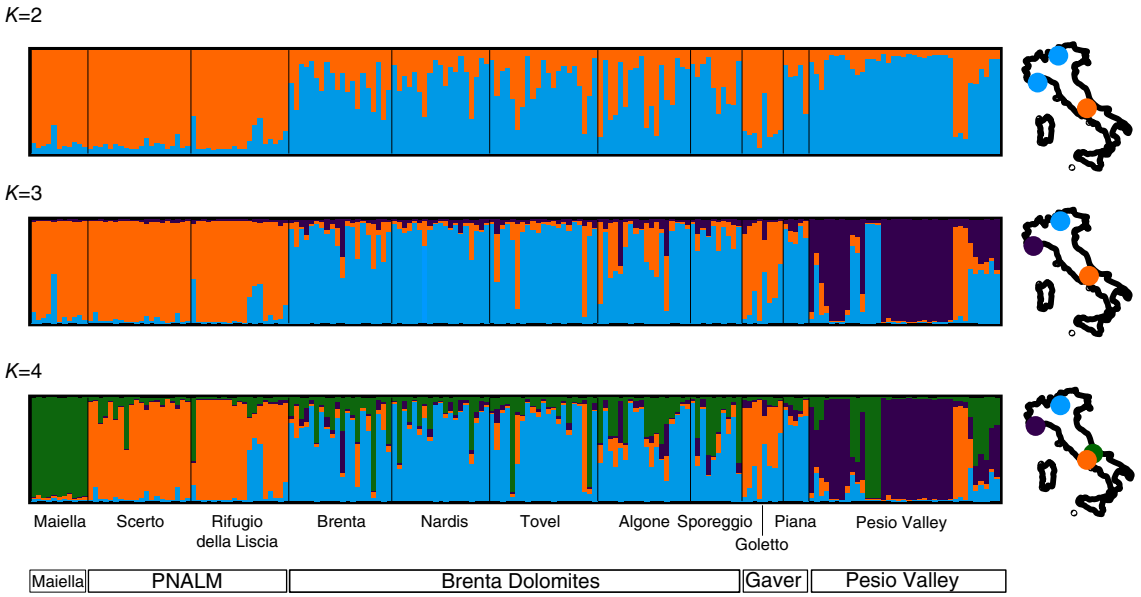

FIGURE 5 Structure bar plot for the individuals of *Cypripedium calceolus* sampled in different Italian populations, considering 2, 3, 4 genetic clusters (*K*). Names underneath the plot refer to the sampling sites and populations as indicated in Table 1. Small maps on the right show the predominant genetic cluster found in each population

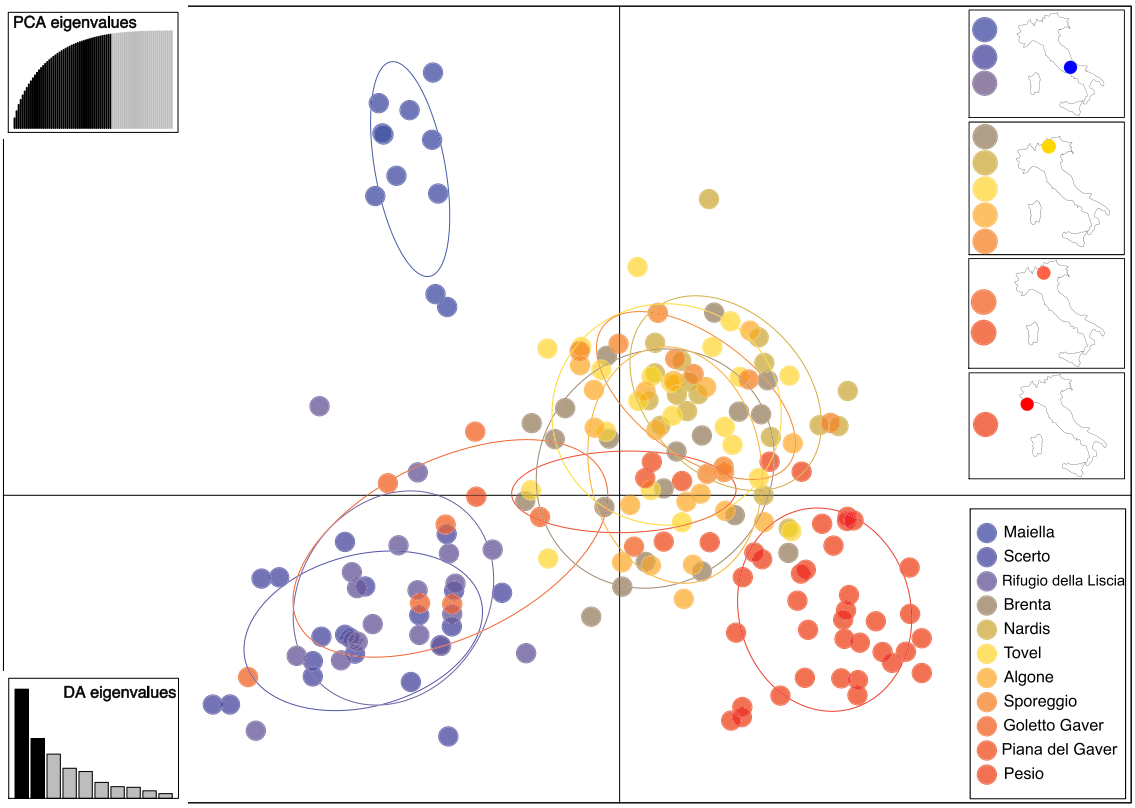

FIGURE 6 Results of the Discriminant Analysis of Principal Components (DAPC) for the individuals of *Cypripedium calceolus* sampled in different Italian populations. Sampling sites in order of appearance in the map are indicated in the legend below

From the Bottleneck analysis, an excess of heterozygotes compatible with a recent reduction in population size was detected in the Maiella population under all the mutation models and tests performed, in combination with a shift in allele frequencies typical of a recent bottleneck (File S2, Figure S5). At PNALM, Brenta Dolomites

and Pesio Valley, only the Wilcoxon test under the IAM model detected an excess of heterozygotes (see details in File S2, Figure S5).

4 | DISCUSSION

4.1 | Genetic diversity and structure in the Italian populations of *C. calceolus*

Our results indicate that genetic erosion threatens the most isolated populations of *C. calceolus* in Italy. Without conservation actions, further isolation and demographic decline will take the populations at Maiella, PNALM and Pesio Valley to the brink of extinction.

The Maiella population is currently the most threatened population, with the lowest values for indices of genetic diversity (both allelic richness and expected heterozygosity) and fixed alleles at two loci. As anticipated from demographic observations, this population has experienced a recent bottleneck and appears as the most differentiated (Figure 6). Remarkably, the Maiella population also shows a negative inbreeding coefficient (Table 1), which is against the expectation of inbreeding/high homozygosity in small, declining populations (Glémin, Bataillon, Ronfort, Mignot, & Olivieri, 2001; Frankham, Bradshaw, & Brook, 2014; see also Angeloni, Ouborg, & Leimu, 2011). This may depend on different factors relating to life-history traits: (1) high mortality of inbred progeny in early life-stages, (2) outcrossing among different genets and (3) persistence of old (long-lived) genets (Brzosko et al., 2002; Husband & Schemske, 1996; Minasiewicz et al., 2018). Regardless of the explanation, such results show that heterozygous individuals may prevail in small populations, but they should not be interpreted as assurance of population fitness.

The populations at Maiella, PNALM and Pesio Valley also display critically low estimates of contemporary effective population size, N_e (Table 1), although the estimate for the Maiella population is likely biased by the small sample size (producing a wide confidence interval for N_e: 95% CI: 2.9–321). The N_e estimate for Pesio Valley is the most precise and reveals an extreme loss of genetic diversity via drift in recent generations (3.1, 95% CI: 2.3–5.1). Low estimates for Maiella, PNALM and Pesio Valley are not surprising, given the declining number of individuals observed (Chung, Nason, & Chung, 2004; Trapnell & Hamrick, 2006; Tremblay & Ackerman, 2001), and should be interpreted as additional evidence for the genetic erosion these populations are facing (Frankham, 2021; Laikre et al., 2021 and references therein).

In terms of genetic structure, the populations at Maiella, PNALM and Pesio Valley appear as separate genetic clusters both in the Structure analysis and DAPC although, except when pairs include the Maiella population, pairwise genetic differentiation is relatively low (<0.2). For example, some individuals at Gaver and PNALM are rather similar (Figures 5 and 6), possibly depending on shared "ancestral" variation, but also on gene flow in the recent past. The relatively high allelic richness and N_e estimated at Gaver despite the small census size also suggest that this population is connected by gene flow to the populations in the Brenta Dolomites, from which it is only weakly differentiated (Figures 5 and 6; File S2, Figure S3).

Caveats for the interpretation of the heterozygosity estimates are associated with the polymorphic nature of microsatellites (Guichoux et al., 2011; Putman & Carbone, 2014; Queirós et al., 2015), potentially leading to inflated heterozygosity values or values which are not comparable across species. In *C. calceolus*, heterozygosity estimates may be considerably high even in threatened populations, depending on the influence of life-history traits explained above. Although useful as a comparative index among infraspecific populations, microsatellite heterozygosity values should not be interpreted as an absolute measure of genetic diversity (Väli, Einarsson, Waits, & Ellegren, 2008). From a comparative perspective, values of genetic variation in the Brenta Dolomites can be used as a baseline for genetic variation in a stable population of *C. calceolus* (Table 1) and, in fact, estimates of genetic diversity in the Brenta Dolomites are also among the highest population estimates in Eurasia (Gargiulo et al., 2019).

4.2 | General guidelines for future conservation actions

Our findings shed light on the genetic diversity in Italian populations of *C. calceolus* and can be integrated with already existing evidence (genetic, demographic and ecological observations) to draw conservation recommendations against the current threats to the species. The most urgent conservation action should be aimed at rescuing the Maiella population (see also Box 2), as the number of mature individuals (~25 ramets) and the genetic results raise a concern about population survival in the short-term. Moreover, we provide general guidelines for practitioners who aim to implement conservation actions in other threatened populations of the lady's slipper orchid in Italy (with a specific appendix in Italian, Appendix S1) and throughout its range. Therefore, we hope to close the gap between science and practice by providing a review of the existing knowledge about the species and by

making genetic results more accessible to the non-academic/non-scientific community.

4.2.1 | Removing the threats to the populations in situ

The first action towards the protection of target populations should be managing or removing the existing threats in situ (Godefroid et al., 2011; Maschinski & Albrecht, 2017). One of the greatest threats to *C. calceolus* is extirpation, especially by people interested in growing *C. calceolus* in their private gardens, and for illegal trade (Hinsley et al., 2018; Jenkins & Oldfield, 1992). In the results of a Population Viability Analysis, Devillers-Terschuren (1999) showed that digging up only one plant every 5 years had serious consequences even in large populations, leaving no chances of survival for a population as small as the Maiella population (<50 individuals). Conversely, projections relating to flower removal were not as alarming, given that *C. calceolus* normally shows low fruit-set and high mortality in early life-stages, although we argue that other unpredictable reproductive constraints may exacerbate the effect of flower removal. Although the risk of random collection cannot be completely ruled out, informing local people, hikers and tourists about (a) the damage the species may undergo even when removing a single flower, and of (b) the extremely low chances of successful cultivation of extirpated plants outside their habitat will certainly raise awareness. Following the project conducted in Switzerland, it was proposed that some of the plants propagated in nursery settings should be made available to the public for a low price, as a strategy to reduce the pressure on wild populations (De Jong & Sprunger, 2020). Unfortunately, this has not been possible under the present national legislation and European Union directives.

Further actions to mitigate the natural threats to the populations have been suggested by Devillers-Terschuren (1999), Kull (1999), and others, and these may involve (1) setting herbivore exclusion cages, which may dissuade both people and herbivores (particularly those digging up rhizomes, such as wild boar); (2) ensuring the occurrence of appropriate light conditions by preventing canopy closure (Hurskainen, Jäkäläniemi, Ramula, & Tuomi, 2017). *Cypripedium calceolus* prefers semi-shaded habitats that pollinators usually visit at the time of the day with most sunlight (Devillers-Terschuren, 1999; Nilsson, 1979). However, clear-cutting which favors the colonization of other competitive species should be avoided (Kull, 1999) because, like many orchids, *C. calceolus* is a primary colonizer of disturbed places, and seedlings prefer sites where competition is low (Cribb, 1997; Perazza & Decarli, 2020).

4.2.2 | Recommendations for plant translocation (reinforcement/reintroduction)

We recommend the reinforcement of the populations at Maiella and PNALM, with the possible introduction of individuals in suitable sites within the range of the two National Parks, aiming to promote gene flow between sites. Such sites must be decided in the field based on the occurrence of the appropriate conditions (substrate and light) and lack of disturbance. Likewise, we recommend population reinforcement in the Pesio Valley, as the population is declining despite being less geographically isolated. If the population size will be increased, gene flow with the nearby populations on the Alps might be promoted/re-established.

As pointed out in previous works, plant translocations may represent the last opportunity to rescue a population, and therefore it should be carefully planned to avoid wasting resources (Albrecht & Edwards, 2020; Fenu et al., 2019; Godefroid et al., 2011; IUCN/SSC, 2013; Novak, Phelan, & Weber, 2021; Van Rossum & Hardy, 2020; Whiteley, Fitzpatrick, Funk, & Tallmon, 2015). For *C. calceolus*, we recommend that practitioners consider the following aspects when planning the (re)introduction of individuals in the wild.

a. *Life-stages for translocation*. Given its complex demography characterized by several life-stages, the choice of the appropriate stage for reintroduction is crucial for the successful establishment of *C. calceolus*.
   The first step in obtaining a progeny for translocation is bypassing the fruit-set limitations typical of *C. calceolus*, by conducting artificial pollinations. Genetic diversity within the progeny can be maximized by selecting as many individuals as possible (but see point [c]), located at spatial distances which minimize the risk of choosing related individuals or clones. Artificial pollinations also guarantee a higher seed production in *C. calceolus*, given that the pollen is directly transferred from one plant to another (De Jong & Sprunger, 2020).
   The highest mortality in terrestrial orchids is associated with the seed, protocorm and juvenile life-stages (Shefferson, Jacquemyn, Kull, & Hutchings, 2020). According to literature reports, individuals of *C. calceolus* remain in their protocorm stage for 3 years before the first leaf appears above ground and enters the juvenile stage (Nicolé et al., 2005 and references therein). However, in optimal conditions such

as nursery settings, germination is much more rapid (De Jong & Sprunger, 2020). Similarly, the age range at which first flowering occurs is reported as 6–10 years (Nicolé et al., 2005; Rasmussen, 1995), but it can be as little as 18 months in seed-raised plants (De Jong & Sprunger, 2020). Therefore, to maximize the reintroduction success, seeds should be germinated in vitro and then reintroduced at a life-stage that widens the chances of survival, ideally the adult stage. Apart from the benefit of discarding weak plants in the early stages of the process (De Jong & Sprunger, 2020), introducing mature individuals during summertime guarantees a quicker response to population decline, as mature individuals would be immediately available for gene flow, and the effect of the translocation intervention may be evident earlier. Moreover, previous experiments suggest that translocating flowering-size plants reduces individual losses due to animal predation.

The availability of resources and infrastructure represents the main constraint to obtaining the appropriate life-stage for reintroduction. For example, large growth chambers may not be available to support individual growth for several years and hundreds or thousands of individuals. However, we stress that producing high-quality material with the appropriate propagation method (Godefroid, Le Pajolec, & Van Rossum, 2016) is especially critical in *C. calceolus*, where even a successful experiment can record 50% losses during propagation (De Jong & Sprunger, 2020). Propagation settings should ensure that the proper phytosanitary conditions are met, to reduce the risk of introducing pathogens together with the translocated plants (IUCN/SSC, 2013; Rossi, Amosso, Orsenigo, & Abeli, 2013).

In the absence of appropriate in vitro facilities, scattering seed from hand-pollinations on bare ground around mature parental plants may successfully increase the population and should be preferred to inaction.

b. *Source population for translocation*. Traditional guidelines recommended selecting source material from genetically related populations growing in similar environmental conditions (McKay, Christian, Harrison, & Rice, 2005). This would allow reducing the risk of outbreeding depression (i.e., the reduction of fitness in the progeny due to the use of excessively differentiated mixed sources; e.g., Edmands, 2007; Frankham et al., 2011, 2017), genetic swamping (i.e., the replacement of local alleles with the newly introduced alleles; e.g., Weeks et al., 2011), and maladaptation, when translocated individuals are not adapted to the recipient environment. However, such guidelines have been deemed as too precautionary and even counterproductive in the case of small and threatened populations (Broadhurst et al., 2008; Maschinski, Wright, Koptur, & Pinto-Torres, 2013; Ralls et al., 2018). Recent studies have demonstrated the potential of a diverse source population for translocation (e.g., Pickup, Field, Rowell, & Young, 2013; Van Rossum, Hardy, Le Pajolec, & Raspé, 2020), which may also result in a wider adaptive variation and ability to respond to local threats (Bucharova et al., 2019).

Three possible alternatives exist for the reinforcement of the Maiella population. The first obvious alternative would be using local individuals, which would remove any chance of outbreeding depression. However, our genetic results show that, even maximizing genetic diversity in the gene pool of the progeny with artificial pollination of diverse parents, most of the original variation in this population is already irreplaceably lost and genetic rescue with non-local sources is necessary to prevent any risk of low fitness in the newly generated progeny. The translocation experiment in Switzerland was conducted using strictly local germplasm due to the fear that gene pools could be swamped by neighboring sites (De Jong & Sprunger, 2020). However, this led to smaller sites (with <50 plants) receiving less vigorous seedlings, casting doubts on their long-term survival and suggesting the need for a compromise in the "local is best" paradigm (e.g., Maschinski et al., 2013).

The second alternative for the Maiella population would be reintroducing individuals from PNALM, which are the most similar both in terms of genetic diversity and environmental requirements. As PNALM is probably facing similar threats in terms of geographical isolation and reduction of the population size, removing potential progeny from this population should be also associated with local reinforcement. These interventions may help re-establish gene flow between the two National Parks in the future. This is the alternative that we recommend, in association with local reinforcement at PNALM, as it minimizes the risk of outbreeding depression and it is easier to achieve as an initial effort to restore the lost genetic diversity.

A third alternative would be to use individuals from the Alpine range, where there is very low risk of reducing the local seed bank. Specifically, we analyzed populations in the Brenta Dolomites, which have high genetic diversity and seem to encompass most of the alleles found elsewhere. This alternative may give interesting results if one of the problems of the Apennine populations is low fitness but may swamp unique genetic diversity that may occur in

the Apennines. Moreover, as we have only considered neutral genetic variation (associated with microsatellite loci), we cannot rule out the risk of outbreeding depression. However, we have found a relatively low genetic differentiation among populations, especially if placed in the context of Eurasian populations (Gargiulo et al., 2019), which may also indicate a low risk associated with mixing locally adapted variants. A further practical limitation associated with this third alternative would be the necessity for concerted action between local institutions and stakeholders in Northern and Central Italy, resulting in further costs and potential barriers. In the Pesio Valley, we recommend population reinforcement using local individuals as the quickest and most efficient strategy. In fact, genetic diversity in this population is still high despite the observed demographic decline and the small effective population size, suggesting that increasing population size may be sufficient to counteract further decline.

c. *How many plants should be translocated?* Reintroducing a limited number of plants may lead to a complete failure of the reintroduction action (e.g., Van Rossum et al., 2020). Current guidelines recommend introducing at least 50 plants in new sites to buffer the expected random mortality (Maschinski & Albrecht, 2017), although the appropriate number of plants to reintroduce also depends on life-history traits and the biology of the species. In *C. calceolus*, factors such as failure to establish a mycorrhizal partner, availability of pollinators, low-fruit set and high-mortality in the early life-stages have also been observed in large, healthy populations (Rasmussen, Dixon, Jersáková, & Těšitelová, 2015), and need to be considered even in the best-case scenario.

Once again, we recognize the compromise between the number of individuals to reintroduce and the available resources/infrastructure. However, reintroducing an insufficient number of plants may jeopardize the experiment or delay the conservation intervention, considering that results are expected in the long-term (see next section; Albrecht et al., 2019) and the expected post-translocation mortality (e.g., Van Rossum et al., 2020). When there is a benefit in splitting costs and resources over the years, a small translocation trial may be attempted in the first year, followed by further translocations stratified over the years. However, the risk of delaying the conservation intervention must be considered.

The degree of isolation and genetic diversity found in the Maiella and PNALM populations, coupled with the general observations about the ecology/biology of the species suggest that genetic erosion and isolation may be only reversed with several hundred individuals. In the Pesio Valley, re-establishing a stable population capable of gene flow and long-term survival also requires several hundred individuals.

Reintroduced individuals should not be planted at high density (<50 cm apart) to avoid competition for space between new ramets generated by clonal growth and new clumps established by seed recruitment.

4.2.3 | Post-translocation monitoring

We recommend establishing a monitoring plan to verify the successful translocation of *C. calceolus*. In particular, the following aspects need to be considered:

a. *Cypripedium calceolus* has a complex demography with life-stages of variable lengths. Therefore, F1 juveniles should not be expected in the year following the translocation, even if adult flowering plants were used. It may take more than a decade to verify whether a self-sustaining population has been established.
b. Individuals can become dormant in the juvenile, young and adult life-stages (Shefferson, Kull, & Tali, 2005), hence some clumps may not appear above ground in a particular year.
c. Habitat or population disturbance may not cause an immediate loss of genetic diversity (delayed genetic erosion), especially because of the long generation time promoted by vegetative reproduction.
d. Post-planting monitoring and plant care are essential, and this can be difficult in remote locations. Clearing the ground, reducing overhead shading and "gardening" the plants (i.e., watering, removing slugs, etc.) are also important.
e. Establishing a "honeypot site" in which planted lady's slipper orchids are displayed for the public, would allow people to visit under supervision without damaging the most sensitive populations (see e.g., Fay & Taylor, 2015).
f. Future genetic monitoring may take advantage of the same set of markers/data we have used in the present study, considering their broad application in Eurasian populations (Gargiulo et al., 2019) and their degree of polymorphism. Even in the long-term, our microsatellite data set may be used as a baseline for comparison, especially in terms of allelic diversity, allowing the consequences of management to be tracked.

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

Our study has provided clear recommendations for the conservation of *C. calceolus*, based on the review of

ecological and population genetic aspects. High-throughput methods targeting many regions in the genome of *C. calceolus* are relatively expensive, given the large genome size of the species (Gargiulo et al., 2021). We recognize that further cost reductions associated with high-throughput methods may allow questions about adaptive variation, in addition to selectively neutral variation (Flanagan, Forester, Latch, Aitken, & Hoban, 2018; Funk, McKay, Hohenlohe, & Allendorf, 2012), to be addressed, but some populations of *C. calceolus* require immediate action, and we cannot afford to wait for such cost reductions.

Unfortunately, there is also uncertainty associated with the future implications of climate change (Geppert et al., 2020; Kramer & Havens, 2009), especially in edge populations, where conditions may become unsuitable for the species. While such uncertainty should be considered in refining strategies (Rusconi, 2017), it should not discourage their implementation in the first place, as populations of *C. calceolus* have primarily disappeared because of human actions, and therefore it is a human responsibility to reverse the damage.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHOR CONTRIBUTIONS

Roberta Gargiulo: conceptualization (lead); writing: original draft (lead); formal analysis (lead); investigation (lead); visualization (lead); writing: review and editing (lead). **Martino Adamo:** resources: sampling of the Pesio Valley population; formal analysis: DNA and microsatellite data for the Pesio Valley population; writing: Appendix S1; writing: review and editing. **Phillip J. Cribb:** conceptualization (supporting); writing: review and editing. **Fabrizio Bartolucci:** investigation: Box 2; writing: Box 2 (lead); writing: review and editing. **Viswambharan Sarasan:** investigation: Box 1 (lead); writing: Box 1 (lead); writing: review and editing. **Claudia Alessandrelli:** investigation: Box 2. **Enzo Bona:** resources: sampling of the Gaver population (lead). **Giampiero Ciaschetti:** resources: sampling of the Maiella population (supporting); investigation: Box 2; writing: review and editing. **Fabio Conti:** investigation: Box 2; writing: review and editing. **Valter Di Cecco:** investigation: Box 2; writing: review and editing. **Luciano Di Martino:** investigation: Box 2; writing: review and editing. **Carmelo Gentile:** resources: sampling of the PNALM population (supporting); investigation: Box 2. **Ana Juan:** formal analysis (supporting); writing: review and editing. **Sara Magrini:** investigation: Box 2; writing: review and editing. **Marco Mucciarelli:** resources: sampling of the Pesio Valley population; writing: review and editing. **Giorgio Perazza:** writing: review and editing. **Michael F. Fay:** funding acquisition for the microsatellite analysis (lead); writing: review and editing.

DATA AVAILABILITY STATEMENT

All genetic data generated and analyzed during this study (for the Italian populations of *C. calceolus*) are included in this published article and associated supplementary information files. Genetic data for the Eurasian populations of *C. calceolus* are available from Gargiulo et al. (2019).

ETHICS STATEMENT

This article does not contain any studies involving human participants performed by any of the authors.

GLOSSARY

adaptive variation	genetic variation associated with reproductive fitness of a population in its environment.
genetic erosion	loss of genetic diversity.
gene flow	movement of genetic variants (alleles) among populations via gametes or migrants. These genetic variants will contribute to the next generation.
genetic rescue	increase in genetic diversity and in reproductive fitness in a small population with reduced genetic diversity, through the introduction of individuals with different/complementary backgrounds.

inbreeding	mating between similar individuals related by descent (including self-fertilization in plants), with production of offspring.
inbreeding depression	reduction in the reproductive fitness of a population due to inbreeding.
reinforcement	human-mediated introduction of individuals into an existing population of conspecific individuals for genetic rescue.
translocation	human-mediated movement of a species, including the reintroduction in an environment in which the species has disappeared, and reinforcement for genetic rescue.
(reproductive) fitness	the number of fertile offspring that survive to reproductive age, contributed by an individual (lifetime reproductive success) (Frankham et al., 2017)

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